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INDUCTION OF THE PAOX1-PROMOTER AND SYNTHESIS OF GFP PROTEIN IN WILD X-33 STRAIN RECOMBINANT *Pichia pastoris* YEAST CELLS

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Recombinant clones of X-33 strain Pichia pastoris *containing the marker gene yEGFP were prepared. The optimal methanol concentration in the medium for induction of heterologous expression was determined in the recombinant clones.*

Key words: *Pichia pastoris,* AOX1-promoter, yEGFP.

The biotechnology of yeast is a very valuable branch of modern industry. Thus, several recombinant proteins have been produced in yeast for pharmacological applications [1]. Most recombinant proteins are synthesized in systems based on type I yeast of *Saccharomyces cerevisiae* and *Pichia pastoris* [2].

The promoter AOX1-gene, which is induced by methanol, is used to prepare recombinant proteins of *P. pastoris*.

Various recombinant proteins that exhibit functional activity are produced in *P. pastoris* cells [3]. The yield of active protein from yeast is known to depend on several factors, the most important of which are:

1) The nature of the heterologous gene and the corresponding properties of the recombinant protein;

2) The genotype of the strain, which sets the base level for expression of the heterologous gene in the cell (genetically determined mechanisms for regulating transcription, translation, splicing, etc.);

3) The morphological features of the strain (structural features of the cell wall and certain organelles) and metabolic characteristics of the yeast cells belonging to various strains;

4) Differences in the genetic stability of recombinant clones of the yeast strains.

The most well studied strain GS115 is auxotrophic for histidine synthesis and was created through mutagenesis [4]. Our investigations are directed mainly at the study of wild yeast strains, in particular, the X-33 strain.

We prepared the genetic construction pPICZB/yEGFP containing the marker gene yEGFP in order to monitor expression of heterologous genes in X-33 strain *P. pastoris* cells [5]. In order to prepare the recombinant yeast vector, we cloned the marker gene yEGFP into yeast vector pPICZB. For this the marker gene was cut from the pUG-36 plasmid at restriction sites for the enzyme XbaI. Vector pPICZB was cleaved from the XbaI site. This produced the linear pDNA vector pPICZB with 3.3 kb and yEGFP with 0.721 kb. These DNA fragments were analyzed in agarose gel with subsequent extraction of the aforementioned fragments from the gel for ligation. Ligation produced recombinant yeast vector pPICZB/yEGFP (Fig. 1). It can be seen that this vector contains the marker gene for the pAOX1-promoter and the gene conferring resistance for the antibiotic Zeocin. After ligation, competent *E. coli* cells were transformed with ligase mixture. Rccombinant clones were selected in medium with added Zeocin. According to genetic maps, plasmid recombinant vector PICZB/yEGFP should have a molecular weight (MW) of 4.048 kb and contain the yEGFP gene in the correct orientation, i.e., the start of the yEGFP gene should be located under the AOX1-promoter. pDNA was isolated from the two *E. coli* recombinant clones collected by us. Restrictive analysis was used to find clones with correct insertion of the yEGFP gene. Restriction analysis was performed by the following enzymes using the scheme:

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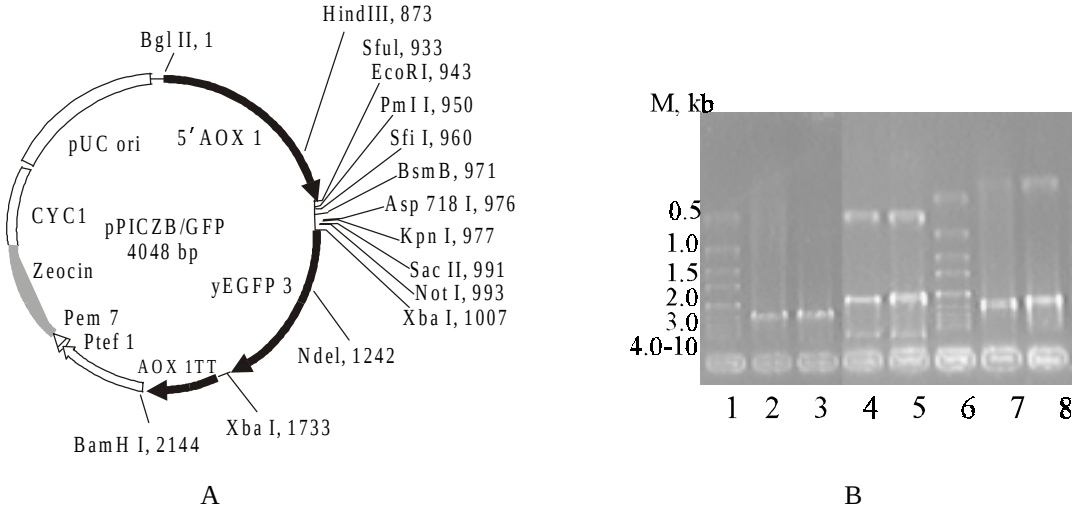

Fig. 1. Map (A) and electrophoretic restrictive analysis (B) of recombinant plasmid pPICZB/yEGFP. 1-kb DNA-marker (1); pPICZB/yEGFP (pDNA clone No. 3, 4) EcoRI-digest (2, 3); pPICZB/yEGFP (pDNA clone No. 3, 4) XbaI-digest (4, 5); DNA mass marker (1-kb DNA-marker) (6); pPICZB/yEGFP (pDNA clone No. 3, 4) Hind III-digest and Nde I-digest (7, 8).

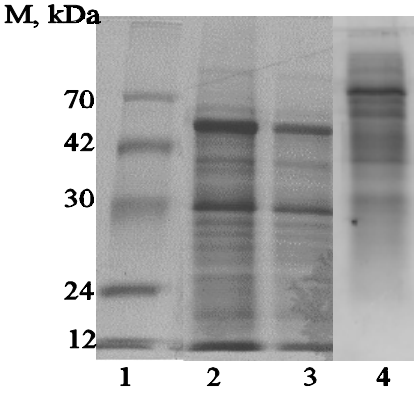

Fig. 2. Electrophoresis of proteins in 12% polyacrylamide gel. Marker proteins (1); suspension of recombinant yeasts X-33/pPICZB/yEGFP-cl.2 (clone No. 2) 72 h after start of induction in methanol (1%) (2); suspension of recombinant yeasts X-33/pPICZB/yEGFP-cl.5 (clone No. 5) 72 h after start of induction in methanol (1%) (3); suspension of recombinant yeasts X-33/pPICZB/yEGFP-cl.5 before methanol induction (4).

1) The MW of pDNA was determined after cleavage by the enzyme EcoRI. The MW of linear pDNA from positive clones should be 4.048 kb.

2) The yEGFP insertion was found using pDNA restriction on XbaI. Positive clones should give a fragment of MW about 0.7 kb.

3) The correct orientation of the insert pDNA was determined by restriction at sites for enzymes HindIII and NdeI. Correct insertation orientation will produce fragments of MW 0.369 and 3.679 kb; incorrect, 0.621 and 3.427 kb. The resulting pDNA fragments were analyzed in agarose gel (1%). Figure 2 shows that pDNA fragments from clones No. 3 and No. 4 have MW corresponding to correct insertion of the yEGFP gene into vector pPICZB. We used *E. coli* No. 3 clones to isolate pDNA pPICZB/yEGFP in preparative quantities.

Then recombinant vector pPICZB/yEGFP was transformed into competent X-33 strain yeast cells. Recombinant yeast clones were collected on YPD medium with Zeocin. Recombinant clones containing the starting pPICZB vector were also obtained as transformation controls and as a determinant of the base expression in yeast.

Further investigations were carried out using three recombinant yeast clones: X-33/pPICZB, X-33/pPICZB/yEGFP-cl.2 (clone No. 2), and X-33/pPICZB/yEGFP-cl.5 (clone No. 5).

The pAOX1 promoter was induced in BMM (Buffered Minimal Methanol) medium. Yeast was precultivated in MGY (Minimal Glycerol) medium [6].

It is known [7] that media containing methanol (0.2-3%) are usually used to induce pAOX1 promoter. The optimal methanol concentration for heterologous expression in recombinant clones of strain X-33 was determined by inducing pAOX1 promoter at concentrations of 0.2, 0.5, 1 and 2%. GFP synthesis was determined using fluorescence and protein electrophoresis in PAAG.

Fluorescence was not found for any of the clones upon induction in medium with 0.2 and 0.5% methanol. However, yeast cells of clones X-33/pPICZB/yEGFP-cl.2 (clone No. 2) and X-33/pPICZB/yEGFP-cl.5 (clone No. 5) cultivated in BMM medium containing 1 or 2% methanol produced fluorescence and synthesized recombinant GFP. The MW of the recombinant GFP was 29 kDa. Synthesis of recombinant GFP protein was determined in 12% PAAG.

Thus, the optimal methanol concentrations in the medium and the time for the start of yEGFP expression were determined using induction of pAOX1 promoter in recombinant clones of *P. pastoris* strain X-33 that synthesize marker protein yEGFP.

EXPERIMENTAL

Construction of the Yeast Vector with the Marker Gene. Cloning used reagents from New England Biolabs. Yeast cells, *E. coli* cells, and the vector were obtained from Invitrogen (USA). Vector pUG-35 was gratefully supplied by Prof. Hagemann (Germany).

Plasmid DNA pPICZB was introduced into competent *E. coli* strain XL2-Blue by electroparation in a 200-μL chamber on a Gene Pulser (Bio-Rad) instrument. The electroparation parameters were potential 2.5 kV, resistance 400 Ω, 0°C, 4.5 ms [8]. Recombinant clones were selected in LB medium (1% yeast extract, 2% peptone, 1% NaCl) with Zeocin (25 mg/mL). pDNA was isolated preparatively from bacteria by alkaline lysis [9]. The resulting pDNA was split by enzyme XbaI in NEB-2 buffer solution (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9). Marker gene yEGFP was isolated from plasmid pUG-36. This plasmd was also amplified preparatively in *E. coli* XL2-Blue and isolated in preparative quantities. The yEGFP gene was isolated by splitting plasmid pUG-36 at sites for XbaI in NEB-2 buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9) and fractionating the resulting DNA fragments in agarose gel (1%) and TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA, pH 7.8). 1-kb DNA marker was used as the marker. A DNA fragment of mass 0.712 kb was extracted from the agarose gel and ligated with the vector using standard methods [9]. Competent E. coli strain XL2-Blue cells were transformed by ligase mixture using electroparation under the conditions described above. Recombinant *E. coli* clones were collected in LB medium with Zeocin (25 mg/mL) [10].

Preparation of Recombinant X-33 Strain *P. pastoris* Yeast Clones. Recombinant yeast clones were obtained by treating yeast cells with LiCl [11]. The competent yeast cells were prepared and recombinant vector pPICZB/yEGFP was transformed using the literature method [12]. Transformed yeast was collected in YPDS medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol) with Zeocin (100 μg/mL).

Induction of Heterologous Expression of Recombinant Yeast Clones. Yeast strains X-33, X-33/pPICZB, X-33/pPICZB/yEGFP-cl.2, and X-33/pPICZB/yEGFP-cl.5 were precultivated in MGY medium [250 mL, 100 mM potassium phosphate, pH 6.0, 1.34% YNB (Invitrogen), biotin (4×10^{-50}), and 1% glycerol]. The yeast suspension was grown until the optical density OD_{600} = 2.0. Then cells were precipitated by centrifugation (5000 rpm, 5 min) under sterile conditions. Heterologous expression was induced in BMM medium [100 mM potassium phosphate, pH 6.0, 1.34% YNB (Invitrogen), biotin (4×10^{-50}), and methanol] [2]. Four flasks for each yeast strain (16 total samples) with BMM medium (50 mL) without methanol were prepared for the induction. Methanol was added to the first four flasks (0.2%); the second four (0.5%); the third four (1%), and to the remaining four (2%). Each yeast strain was resuspended in four flasks with 0.2, 0.5, 1, and 2% methanol, respectively until OD_{600} = 0.1. The fluorescence of the yeast suspension at 365 nm was determined each 4 h. The growth rate of the induced

yeast culture was determined from the change of optical density of the suspension (OD_{600}) 12, 24, 36, 48, 60, 72, 84, 96, and 108 h after the start of induction. Samples for protein electrophoresis in PAAG (12%) were collected 48, 60, and 72 h after the start of induction by methanol. Synthesis of recombinant GFP protein was observed 72 h after the start of induction.

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